ELSEVIER

Journal of Chromatography B, 737 (2000) 203–212

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Purification of surface-associated urease from *Helicobacter pylori*

Elmar Rokita*, Athanasios Makristathis, Alexander M. Hirschl, Manfred L. Rotter

Department of Clinical Microbiology, Hygiene-Institute, University of Vienna, Währinger Gürtel 18-20/5P, A-1090 Vienna, Austria

Abstract

Helicobacter pylori colonizes the human gastric mucosa and produces large amounts of urease. The enzyme was extracted from the bacteria by distilled water and purified by gel-permeation (Sephacryl S-300), anion-exchange chromatography (Mono Q) and a second gel-permeation (Superdex 200). Urease enzyme activity was detected with a spectrophotometic assay based on phenol red. The optimal pH for anion-exchange was 6.9. The recovery of urease was 55–75%, purity 93–98% and the overall protein recovery 0.8–1.4%. The urease in the final extract still had enzymatic activity and showed the typical subunits of M_r 66 000 and M_r 30 000 when subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Keywords: Purification; *Helicobacter pylori*; Enzymes; Urease

1. Introduction

The Gram-negative bacterium *Helicobacter pylori* colonizes the human gastric mucosa and is associated with several gastric disorders including carcinoma [1]. Among the virulence factors characterized until today is an urease of M_r 380 000, which helps the pathogen to survive in the acidic environment of the stomach [2,3]. In addition, urease is involved in chemotactic reactions of the bacterium and reduces opsonization by human complement [4,5]. The enzyme consists of six subunits UreA (M_r 30 000) and six subunits UreB (M_r 66 000) [6]. Unlike other bacterial ureases the enzyme is found not only in the cytoplasma of the cell but also on the bacterial surface [7,8]. The pathway by which urease becomes surface-associated is still under investigation.

The purification of urease from *H. pylori* has been described in a number of publications in the past [2,6,8,9]. But none of these protocols was designed for purifying large amounts of enzymatically active urease routinely. Using the highly integrated chromatographic system ÄKTA*purifier* (Pharmacia Biotech, Uppsala, Sweden) we aimed to establish a simplified high yield urease purification protocol delivering approximately 1 mg of protein per run. The recovered protein should retain enzymatic activity and should be pure enough (90–95%) for stimulation experiments with a panel of different cells or cell lines.

2. Experimental

2.1. Chemicals

The chemical supplier's name is given in brackets the first time it is mentioned in the text. If no

*Corresponding author. Tel.: +43-1-40400-5158; fax: +43-1-40400-5162.
E-mail address: elmar.rokita@akh-wien.ac.at (E. Rokita)

0378-4347/00/$ – see front matter
PII: S0378-4347(99)00374-6

company is specified the chemical was purchased from Merck, Darmstadt, Germany. Water was obtained from our laboratory distillation facility.

2.2. Bacterial culture

H. pylori was grown on Columbia agar with 5% sheep blood (Becton Dickinson, Meylan, France) at 37°C under microaerobic conditions (85% N_2, 5% O_2, 10% CO_2; Heraeus Cytoperm, Osterode, Germany).

2.3. Extraction of urease

Twenty agar plates were inoculated with a dense suspension of *H. pylori* N6 in sterile 0.9% NaCl. After 96 h of incubation the confluent layer of bacterial cells was harvested with a cell scraper (Greiner, Kremsmünster, Austria) and collected in a SE-12 tube (Sorvall, DuPont, Wilmington, DE, USA). An 8-ml volume of iced distilled water was added and the tube was vortexed vigorously (2500 rpm) for 1 min. After centrifugation (Sorvall RC5C; 25 000 *g*, 10 min, 4°C) the supernatant was recovered, mixed with 1/10 volume of 10× gel-permeation buffer [1×: 50 m*M* NaH_2PO_4, 150 m*M* NaCl, 1 m*M* EDTA (Bio-Rad, Hercules, CA, USA), adjusted to pH 7 with solid NaOH], filtered through a 0.22-μm syringe filter unit (Millipore, Molsheim, France) and concentrated to approximately 4 ml in a stirred ultrafiltration cell.

2.4. Chromatographic instrument

A fast protein liquid chromatography (FPLC) system Model ÄKTA*purifier* with UV, conductivity and pH sensors was employed. The whole system was placed in a cold laboratory (Pharmacia) and all separations were carried out at 16°C. Proteins were detected by absorbance at 280 nm. Temperature and pressure was recorded routinely. The system was controlled by Unicorn software version 2.30 (Pharmacia) installed on a personal computer. Samples were injected using prefilled wire-loops or a 10-ml super-loop (Pharmacia). Fractions were collected automatically either continously or according to UV peaks.

2.5. Column chromatography

Three subsequent chromatographic separations were established. Initially, the concentrated water extract was subjected to a Sephacryl S-300 HR 26/60 column (60×2.6 cm I.D., Pharmacia) equilibrated with gel-permeation buffer. The flow-rate was 1 ml/min and 4-ml fractions were collected continously after V_0 was reached. After screening the fractions for urease, the contents of positive tubes corresponding to a single peak were pooled, dialyzed against ion-exchange loading buffer {20 m*M* 1,3-bis[tris(hydroxymethyl)-methylamino]propane (Bis-Tris propane) (Sigma, St. Louis, MO, USA) adjusted to pH 6.9 with HCl} using a stirred ultrafiltration cell and concentrated to approximately 3 ml. In the next step anion-exchange chromatography was performed using a Mono Q HR 5/5 column (5×0.5 cm I.D., Pharmacia) equilibrated with the same buffer. The flow-rate was 1 ml/min and UV peaks were collected during elution with a linear NaCl-gradient (0–500 m*M*, 18 ml). After screening the fractions for urease, the contents of positive tubes corresponding to a single peak were pooled, dialyzed against gel-permeation buffer and concentrated to 0.5 ml. Subsequently, the material was loaded onto a Superdex 200 HR 10/30 column (30×1 cm I.D., Pharmacia) equilibrated with gel-permeation buffer. The flow-rate was 0.5 ml/min and UV peaks were collected. Finally, urease-positive fractions were concentrated to approximately 1 ml.

2.6. Dialysis and concentration

A 10-ml stirred ultrafiltration cell Model 8010 containing a M_r 30 000 cut-off membrane and connected to an N_2 gas supply and a pressure-resistant reservoir was employed (Amicon, Beverly, MA, USA). Dialysis was considered 99% complete when five-times the volume of the sample had passed through the membrane [10]. To concentrate small samples simultaneously disposable filter units Centricon-10 (Amicon) were also used.

2.7. Determination of protein content

The standard colorimetric method according to Bradford was used [11]. A 10-μl volume of sample

(if necessary diluted with 0.9% NaCl) were mixed with 990 μl of Bradford solution [20 mg Coomassie Brilliant Blue G-250 (Serva, Heidelberg, Germany) was dissolved in 10 ml of 95% ethanol, 20 ml of 85% phosphoric acid was added and the volume was adjusted to 200 ml with distilled water] and the absorbance was read at 595 nm after 2 min in a spectrophotometer (Hitachi U2000, Tokyo, Japan). A 10-μl volume of 0.9% NaCl served as blank. Bovine serum albumin (BSA) (Sigma) in the range of 0.125 to 20.0 μg/ml was utilized to generate a standard curve.

2.8. Urease-screening

After each column chromatography 20 μl of each fraction were mixed with 300 μl of testing buffer (3 m*M* NaH_2PO_4, 110 m*M* urea, 7 μg/ml phenol red, adjusted to pH 6.8 with NaOH, sterifiltered [6]) in a 96-well flat bottom microtiter plate (Nalge Nunc, Rochester, NY, USA) using a multichannel pipette to minimize handling delay. Blank wells received 20 μl chromatography buffer. The plate was immediately covered with a sticky seal and incubated at 37°C. In wells containing urease the color slowly changed from yellow to pink. The absorbance at 560 nm was quantitatively determined in a microplate reader (Dynatech MR7000, Guernsey, UK) by serial measurement at different time points up to 20 min.

2.9. Determination of urease specific activity

To quantify urease activity more precisely 600 μl of testing buffer were placed in a 1-ml semi-micro cuvette (Brand, Wertheim, Germany) and the absorbance was set to zero in a spectrophotometer at 560 nm. Without removing the cuvette 5–50 μl of the sample was added and mixed immediately. With a maximum delay of 15 s the absorbance was determined sequentially every 6 s during a period of 3 min. If the change in absorbance (ΔA) was <0.2 the assay was repeated with an increased volume of sample. Absorbance was plotted against time and $\Delta A/t$ was calculated from the linear part of the curve. The corresponding amount of ammonia was assessed by a serial dilution of a purchased 25% ammonia solution. After determination of the protein content the specific activity (m*M* ammonia per mg protein per min) could be calculated.

2.10. Electrophoresis

Continuous denaturing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) according to Laemmli was used to determine the grade of purification [12]. The gel stock solution, which contained 33.5% acrylamide and 0.3% *N*,*N*′-methylenebisacrylamide (Amresco, Solon, OH, USA) was sterifiltered and stored at 4°C. A 72×82×0.75 mm gel [4% stacking (310 m*M* Tris; USB, Cleveland, OH, USA, adjusted to pH 6.8 with HCl, 0.1% SDS; Amresco, 0.05% ammonium persulfate {APS}; Amresco, 0.1% *N*,*N*,*N*′,*N*′-tetramethylethylenediamine {TEMED}; Bio-Rad) and 12% separation gel (380 m*M* Tris–Cl, pH 9.1, 0.1% SDS, 0.075% APS, 0.05% TEMED)] with 10 lanes was purified and set up in a mini-Protean II cell (Bio-Rad) and run at 200 V for 50 min. The following buffers were used: 125 m*M* Tris–Cl, pH 6.8, 20% glycerol, 4% SDS, 2% 2-mercaptoethanol, 0.1 mg/l bromophenol blue (Sigma; 2× sample buffer); 25 m*M* Tris–Cl, pH 8.3, 200 m*M* glycine, 3.5 m*M* SDS (running buffer). Denaturation of proteins was done by diluting the samples with sample buffer (1:2) and heating the mixtures at 75°C for 20 min. After electrophoresis, gels were stained in staining solution (10% acetic acid, 25% isopropanol, 0.5 g/l Coomassie Brilliant Blue G-250) for 30 min and destained in 10% acetic acid over night. Standard proteins served as markers for molecular mass (LMW-kit, Pharmacia). Densitometry was performed with a gel documentation system and the RFLPscan plus software version 3.12 (MWG-Biotech, Ebersberg, Germany). Gels were mounted on Whatman-3 (Whatman, Clifton, NY, USA) filter paper, dried (gel dryer 2003, Pharmacia) and stored for permanent record.

2.11. Western blotting

After SDS–PAGE of samples using a 10% separation gel (52×82×0.75 mm) the proteins were electroblotted (0.8 mA/cm^2, 1 h) onto nitrocellulose (Hybond-C 0.45 μm, Amersham, Little Chalfont, UK) employing a Multiphor II NovaBlot semidry-unit (Pharmacia). Transfer buffer contained 26 m*M*

Tris–Cl, pH 8.3, 192 m*M* glycine and 20% methanol [13]. The protein transfer was verified by staining the gel with Coomassie Brilliant Blue and the membrane with Ponceau S [0.2% Ponceau S (Sigma), 3% trichloroacetic acid, 3% sulfosalicylic acid] [14]. After destaining in distilled water the membrane was blocked by an over night incubation in Blotto [Tris-buffered saline with Triton X-100 (TBS-T) (50 m*M* Tris–Cl, pH 7.4, 137 m*M* NaCl, 5 m*M* KCl, 0.1% Triton X-100; Serva) supplemented with 5% nonfat dry milk (Agrana, Gmünd, Austria) and 10 m*M* Na-azide] at 4°C [15]. Polyclonal rabbit antibodies against recombinant UreA and UreB maltose binding protein (MBP)-fusion proteins provided by Dr. H. DeReuse (Pasteur Institute, Paris, France) were used to detect *H. pylori* urease subunits. The membrane was incubated with 15 ml of an antibody dilution in Blotto (anti-UreA 1:100 000 and anti-UreB 1:10 000, respectively) for 2 h at room temperature with constant agitation and washed three times in TBS-T. Primary antibodies were detected by incubating the membrane in 15 ml of goat anti-rabbit–IgG horse-radish peroxidase conjugate (A545; Sigma) diluted 1:10 000 in TBS-T for 45 min at room temperature with constant agitation. After four washing steps with TBS-T the blot was immersed in 2 ml (solution 1+2) ECL-Western blotting detection reagents for 1 to 2 min, a piece of Hyperfilm ECL was exposed in a Hypercassette for 20 s (all from Amersham) and the film was developed.

3. Results and discussion

The enzyme activity of *H. pylori* urease is critical for the survival of the bacterium and colonization of the gastric epithelium. In contrast to other bacterial ureases, *H. pylori* urease is in part surface-associated. The bacterium could also take advantage of other functions of urease (e.g., reduction of opsonization), which are not dependent on enzyme activity. It is likely that these functions are caused rather by extracellular than intracellular urease since direct contact is a prerequisite in the process. The pathway by which urease is translocated across the cytoplasmic membrane is poorly understood. Although a passive adsorption mechanism after autolysis of aged cells is proposed in the literature [16], there may also exist a yet undiscovered active transport mechanism involving for example typ I secretion pathways [17]. Since urease could be modified in some way during transport we intended to focus on surface-associated urease for the investigation of specific reactions of cells or cell lines in response to urease. In this context a relatively simple purification protocol for surface-associated urease with high relative and absolute yield is needed.

Available purification protocols do not meet these criteria. Dunn et al. [2] used a Superose 12 HR 10/30 column as primary purification step, which has a suboptimal separation range of M_r 10 000–300 000 and does not tolerate more than 240 μl of sample. Concentration of a water extract from 20 agar plates with confluent bacteria to this volume is virtually impossible because of extremely high viscosity and partial precipitation (our observation). Moreover, the enzyme recovery was only 13.3%. Hu and Mobley [6] employed a French press to lyse the bacteria, which makes it impossible to distinguish between surface-associated and intracellular urease, used Phenyl-Sepharose in the second (of overall 4) purification step, although 43% of the urease loaded did not bind to the resin and published a final enzyme recovery of 12.2%. Hawtin et al. [8] lysed the bacteria by sonification, applied a Superose 6 HR 10/30 column (maximal loading volume 240 μl) and continued with a non-denaturing PAGE, which is time-consuming (19 h). Unfortunately, enzyme recovery is not cited. Finally, Austin et al. [18] utilized sequential gel-permeation (Superose 12 HR 10/30) runs resulting in only 5.5% enzyme recovery.

In preliminary experiments it could be shown that about 75% of urease could be extracted from bacterial cells by a brief suspension in distilled water (data not shown). Rest activity remained associated with the cell pellet and could be due to cytoplasmic urease since approximately the same proportion of whole cell urease activity (75%) could be inhibited by 1 μ*M* of the poorly diffusable urease inhibitor flurofamide (Tocris, Bristol, UK), which inhibits only surface-associated urease activity [7]. This result together with the fact that extracted cells remain viable indicated that surface-associated urease extraction by distilled water is sufficient without leading to lysis of the bacterial cell. At this stage we also tested the extraction of urease by the

anionic detergent *n*-octyl β-D-glucopyranoside (1%; Sigma) as proposed by Evans et al. [9]. Since this treatment led to significant cell lysis the distilled water urease extraction protocol was preferred. However, extraction with distilled water should not exceed 1 min. In order to avoid that bacteria dry out and form tight clumps, which would deteriorate suspension in water, they should be harvested in a short time. In this context cell scrapers originally designed for handling cell cultures turned out to be a useful tool to harvest confluent bacteria quickly and quantitatively from a lot of culture plates. The inclusion of reducing agents like 2-mercaptoethanol or phenyl-methyl-sulfonyl-fluoride (both 1 m*M*; Sigma) in the extraction step was not appropriate since the recovered urease activity declined. In contrast to water, phosphate buffered saline or a liquid culture medium [Brucella broth (Difco, Detroit, MI, USA) +5% heat-inactivated fetal calf serum (Gibco BRL Life Technologies, Paisley, UK)] failed to extract surface-associated urease.

After the water extract was adjusted to gel-permeation running buffer by adding a concentrated stock solution a precipitate was visible. This material contained protein as indicated by a Bradford test but no urease as assessed by activity measurement before and after addition of the stock solution.

The supernatant was subjected to size-exclusion chromatography on a Sephacryl S-300 HR 26/60 column, which has a separation range of M_r 10 000–1 500 000, a V_0 of 114 ml and tolerates up to 13 ml of sample. One of the major advantages of our method is the possibility to load high sample volumes without losing resolution. The chromatogram in Fig. 1 starts with the injection of the sample, shows the absorbance at 280 nm (solid line) and the maximum urease activity indicated by the dashed line was found in the third peak (122.02 ml retention) representing 6.2% of the total peak area. Fractions pooled for further fractionation are indicated by the bar. The other peaks obviously representing many different proteins were not further characterized.

After dialysis against anion-exchange buffer and concentration, the material was loaded on a Mono Q HR 5/5 column. After starting a linear NaCl-gradient from 0 to 500 m*M* (500 m*M* to 1 *M* represents only a cleaning phase; straight line) protein elution was followed by absorbance at 280 nm (solid line, Fig. 2). The chromatogram starts with the injection

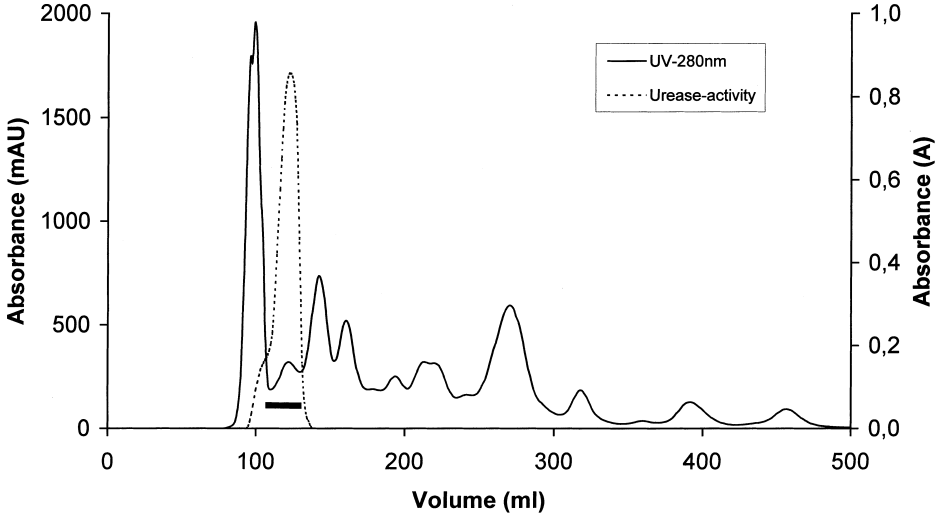

Fig. 1. Size-exclusion chromatography of *H. pylori* proteins on a Sephacryl S-300 HR 26/60 column (60×2.6 cm I.D.) equilibrated with running buffer (50 m*M* NaH_2PO_4, 150 m*M* NaCl, 1 m*M* EDTA, pH 7) at 1 ml/min and 16°C. A 4-ml volume of water extracted proteins was applied to the column after adjusting the salt concentration to running buffer. Proteins were detected by absorbance at 280 nm (mAU, solid line), urease activity was determined in an aliquot of the elution fractions (4 ml) semiquantitatively by a spectrophotometric assay based on phenol red (A, dashed line). Bar=fractions pooled for further purification.

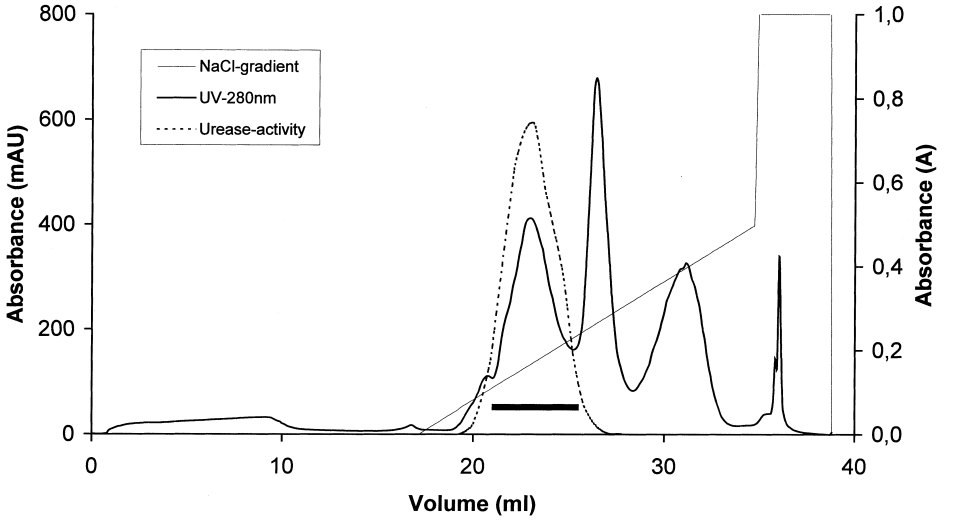

Fig. 2. Anion-exchange chromatography of urease-positive Sephacryl-fractions on a Mono Q HR 5/5 column (5×0.5 cm I.D.) after dialysis against 20 m*M* Bis–Tris propane–HCl buffer, pH 6.9 and concentration to 3 ml. The column was equilibrated and run with the same buffer at 1 ml/min and 16°C. Elution of bound proteins was achieved by a linear NaCl-gradient (0–500 m*M*, 18 ml; straight line) in the same buffer [the second gradient step (1 *M*) represents a cleaning phase]. Proteins were detected by absorbance at 280 nm (mAU, solid line), urease activity was determined in an aliquot of the elution fractions semiquantitatively by a spectrophotometric assay based on phenol red (A, dashed line). Bar=fractions pooled for further purification.

of the sample and maximum urease activity (dashed line) was found in the first major peak (22.90 ml retention) representing 36.8% of the total peak area. As judged by conductivity measurement urease eluted at an average of 12 to 13 mS/cm. Fractions pooled for further fractionation are indicated by the bar. The peaks on both sides of the urease-peak were characterized by SDS–PAGE. While the leading peak consisted of three proteins or their subunits in similar amounts the following peak represented one major protein of M_r 55 000 and nine others including traces of UreA and UreB. The pH of the anion-exchange buffer was tested thoroughly in the first isolation procedures. Tris-2,2-bis-(hydroxyethyl)-(iminotris)-(hydroxymethyl)methan (Bis-Tris) buffers of different pH were created online by immersing the 4 pump inlets in water, 50 m*M* Tris–70 m*M* Bis-Tris, 2 *M* NaCl, and 0.1 *M* HCl. The pH was then scouted in 0.25-steps from 6.5 to 7.75 and the resolution of urease was monitored. The effective concentrations of Tris and Bis-Tris during the run are about 25 m*M* and 35 m*M*, respectively. For better comparison an overlay of these chromatograms is presented in Fig. 3. The urease-peak is marked by an arrow. As 6.9 turned out to be the most convenient pH value, we used Bis-Tris propane buffer because of its pH range.

Finally, the last chromatogram was generated by subjecting the dialyzed and concentrated remaining material to another size-exclusion chromatography with Superdex 200 HR 10/30 (Fig. 4). At this stage concentration of the sample to ≤0.5 ml is possible due to the reduced protein concentration. The absorbance at 280 nm indicates two major (8.48 ml and 9.55 ml retention; V_0=7.77 ml) and a few contaminating peaks. Urease activity correlated with the major two proteins (dashed line) representing 80.1% of the total peak area. The fractions marked with the bar were concentrated and stored. It was surprising that urease was obviously present in two high-molecular-mass peaks very close to each other (termed 1 and 2 in Fig. 4). To assess whether a contaminating protein was responsible for that phenomenon, we subjected the peaks to SDS–PAGE separately, but both lanes showed only the typical urease subunits (data not shown). Therefore we assume that this phenomenon is caused by aggregation of urease, which is in concert with the literature (M_r 380 000–

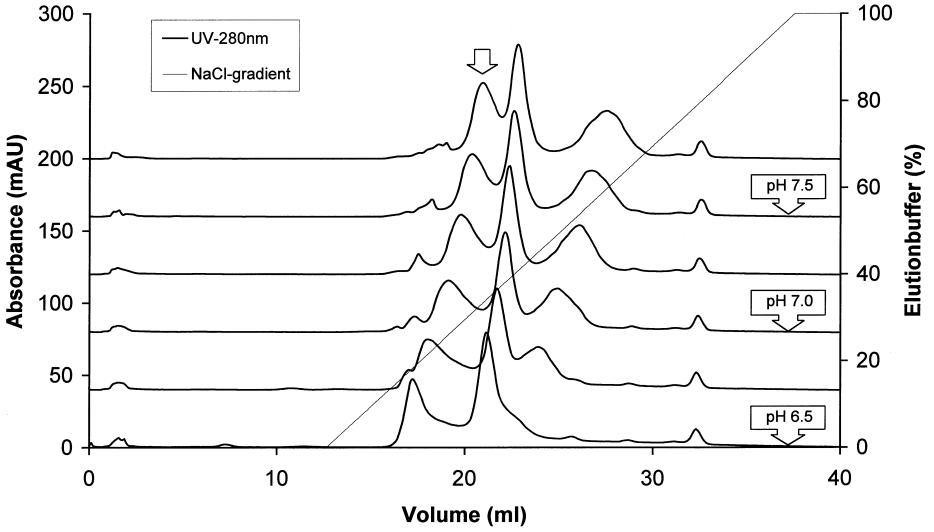

Fig. 3. Overlay of six subsequent anion-exchange chromatograms of 500-μl aliquots of a pool of urease-positive Sephacryl-fractions on a Mono Q HR 5/5 column (5×0.5 cm I.D.) equilibrated with 25 m*M* Tris–35 m*M* Bis-Tris–HCl buffer of pH 6.5 to pH 7.75 in 0.25 steps. Sephacryl fractions were dialyzed against the same buffer with a pH of 7.5 prior to injection. The flow-rate was 1 ml/min at 16°C and elution of bound proteins was achieved by a linear NaCl-gradient (0–1 *M*, 25 ml; straight line). Proteins were detected by absorbance at 280 nm (mAU, solid lines) and the peak containing the maximum urease activity is marked by an arrow.

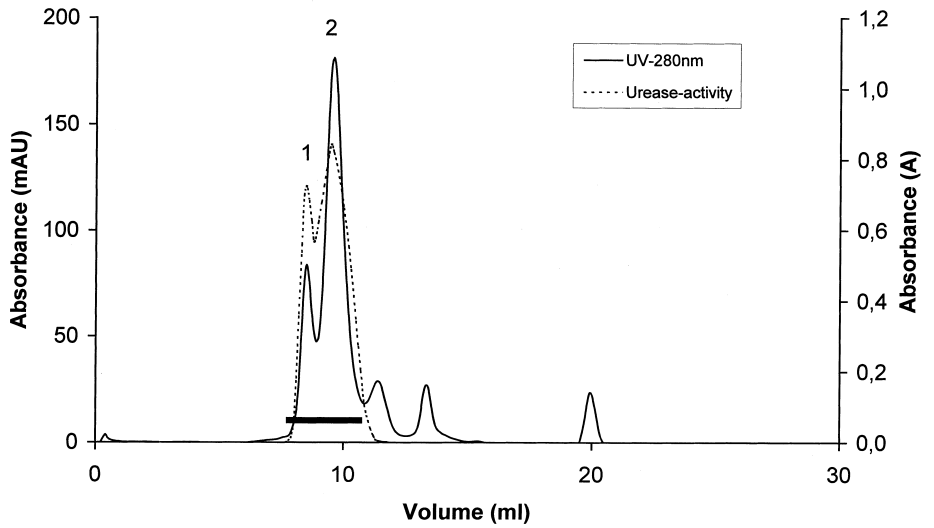

Fig. 4. Size-exclusion chromatography of 250 μl of a pool of urease-positive Mono Q fractions on a Superdex 200 HR 10/30 column (30×1 cm I.D.) equilibrated with running buffer (50 m*M* NaH_2PO_4, 150 m*M* NaCl, 1 m*M* EDTA, pH 7) at 0.5 ml/min and 16°C. Mono Q fractions were dialyzed against the same buffer. Proteins were detected by absorbance at 280 nm (mAU, solid line), urease activity was determined in an aliquot of the elution fractions semiquantitatively by a spectrophotometric assay based on phenol red (A, dashed line). Bar=fractions pooled, representing urease of >93% purity.

680 000 is reported, [18]). The fact that Superdex 200 has a separation range of M_r 10 000–600 000 also supports this assumption.

After each column chromatography, an aliquot of the fractions selected for further separation as well as aliquots of the whole bacteria and the water extract samples were characterized by SDS–PAGE (Fig. 5). Throughout the purification protocol proteins of approximately M_r 30 000 and M_r 66 000 – representing the urease subunits UreA and UreB – become more and more predominant reaching up to 98% of the total protein in the end as judged by scanning densitometry. Since resolution was generally better with lower protein-input the final purity was about 93% when bacteria from 20 confluent culture plates were extracted and rose to approximately 96% when only 10 plates were used. When performing the protocol with low protein-input, purity exceeding 92% could already be achieved after the anion-exchange chromatography. Compared to the literature this represents the shortest purification strategy.

Samples of protein from several different purification runs were subjected to SDS–PAGE. In some of the gels two proteins very close to each other were visible at approximately M_r 66 000 (Fig. 5). To test whether these proteins both represent UreB a Western blot was conducted. Both lanes could be stained by a specific polyclonal antibody against UreB (Fig. 5). Therefore, we assume that protease-digestion might be responsible for this observation. As expected, the protein with M_r 30 000 stained well with a specific polyclonal antibody against UreA (Fig. 5).

As compared with whole cell protein of the bacteria the water extract lacked some high-molecular-mass proteins (Fig. 5).

After each column chromatography the protein content and the urease activity of the fractions selected for further separation was assessed after they were dialyzed and/or concentrated. On the basis of these data, protein recovery and specific activity was calculated. Table 1 gives a summary of these data from a representative run. Starting with about 52 mg total protein 0.488 mg pure urease or 0.94% of the total protein was recovered in the end. Since

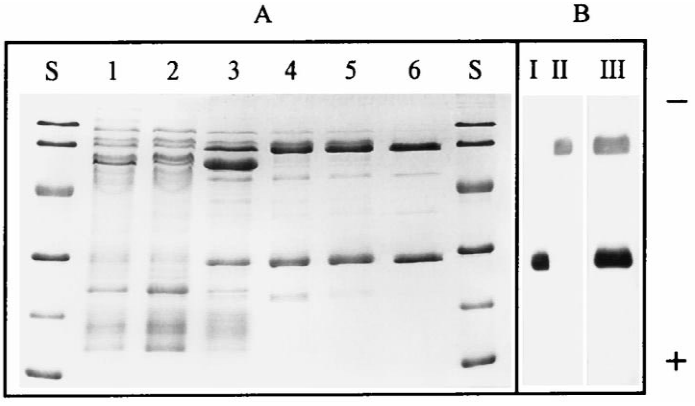

Fig. 5. Characterization of proteins in aliquots of pooled urease-positive fractions after column chromatography or from aliquots of *H. pylori* and the initial water extract: (A) SDS–PAGE using a 12% separation gel, (B) Western blotting after SDS–PAGE using a 10% separation gel. Denaturation of proteins was done by diluting the samples (adjusted to ~2 mg/ml) with sample buffer (1:2) and heating the mixtures at 75°C for 20 min. A 10-μl volume was loaded on the gel and electrophoresis was done at 200 V for 50 min. The gel was stained in staining solution (10% acetic acid, 25% isopropanol, 0.5 g/l Coomassie Brilliant Blue G-250) for 30 min and destained in 10% acetic acid over night. S=Low-molecular-mass standard (3.5 mg/ml, 3 μl per lane): phosphorylase-b M_r 94 000, bovine serum albumin M_r 67 000, ovalbumin M_r 43 000, carbonic anhydrase M_r 30 000, soybean trypsin inhibitor M_r 20 100, α-lactalbumin M_r 14 400. Lanes: 1=*H. pylori* whole cell, 2=water extract, 3=pooled urease-positive fractions after Sephacryl, 4 and 6=pooled urease-positive fractions after Mono Q, 5=pooled urease-positive fractions after Superdex. Proteins in lanes 1–5 represent material from a ''high-input'' purification, proteins in lane 6 from a ''low-input'' purification (for explanation see Results). Two aliquots from pooled urease-positive fractions after Mono Q were electrophoresed separately and blotted onto nitrocellulose. One lane was cut in two pieces and probed with anti-UreA (1:100 000, I) and anti-UreB (1:10 000, II) separately, the other lane with a combination of both antibodies (III). An appropriate horseradish peroxidase labeled secondary antibody and commercially available detection kit was used to develop the blot.

Table 1
Column chromatography of urease from *H. pylori*[a]

	Protein concentration (mg/ml)	Volume (ml)	Protein (mg)	Recovery (%)	Total recovery (%)	Specific activity (m*M* urea/mg protein/min)
Water extract	12.2	4.50	54.9 (51.85)	100	100	1.803
After Sephacryl	1.50	4.20	6.30 (5.75)	12.15	12.15	3.445
After Mono Q	2.40	0.65	1.56 (1.15)	27.15	3.01	10.196
After Superdex	2.22	0.22	0.488	42.40	0.94	26.036

[a] Protein was extracted from *H. pylori* by a brief suspension in distilled water. Protein concentration was determined by the Bradford method with BSA as a standard. The amount of protein in parentheses is the amount which was actually subjected to the next column. By minimizing the difference the final total recovery can be increased to ~1.4%. Recovery means the amount of protein recovered from column-to-column. Total recovery means the amount of protein recovered compared to the extracted amount. Urease activity per min was determined by a spectrophotometric assay based on phenol red from the linear part of the curve. These values were compared with an ammonia standard curve and divided by 2 since one molecule of urea gives rise to two molecules of ammonia when hydrolyzed. Finally, the values were normalized for protein content.

several samples were drawn for documentation purposes during this particular preparation the recovery is at the lower range of the method's potential. More quantitative application of the protein solutions resulted in recoveries up to 1.4% of the total protein. Given the fact that urease represents about 1.8% of the protein in the water extract (based on peak-integration data) the recovery of urease using our method is quite high: the water extract from bacteria harvested from a total of 20 agar plates contains approximately 55 mg protein and 1.0 mg urease of which about 75% can be recovered. Thus we established a *H. pylori* urease purification protocol, which is fast and easy to perform, with high reproducibility in terms of purity and yield, and, therefore, suitable for routine application.

4. Conclusion

Existing purification protocols for *H. pylori* urease deliver only small amounts of pure urease either because of low enzyme recovery (<15%) or due to the inability to load large amounts of sample onto the primary columns. Therefore, we developed a three-step purification protocol starting with a size-exclusion column Sephacryl S-300 HR 26/60, which allows high amounts of protein (<100 mg) and volume (<13 ml). The anion-exchange chromatography used as second step (Mono Q HR 5/5) was optimized and run at pH 6.9. After the third step (Superdex 200 HR 10/30) urease purity was ~95% and the enzyme recovery was 50–75% (equating with a total protein recovery of 0.9–1.4%). High yield, good purity, low time consumption and implementation of an automated chromatographic system are the major advantages of our method.

Acknowledgements

We thank Dr. A. Labigne (Pasteur Institute, Paris, France) for providing *H. pylori* strain N6 and Dr. H. DeReuse (Pasteur Institute, Paris, France) for offering polyclonal anti-UreA and anti-UreB antibodies. We further thank C. Altmutter for her practical help and Dr. A. von Gabain and Dr. T. Decker for useful discussions (Institute of Microbiology and Genetics, University of Vienna, Austria).

References

[1] R.J. Hopkins, J.G. Morris, Am. J. Med. 97 (1994) 265.
[2] B.E. Dunn, G.P. Campbell, G.I. Perez-Perez, M.J. Blaser, J. Biol. Chem. 265 (1990) 9464.
[3] B.J. Marshall, L.J. Barrett, C. Parkash, R.W. McCallum, R.L. Guerrant, Gastroenterology 99 (1990) 697.
[4] H. Nakamura, H. Yoshiyama, H. Takeuchi, T. Mizote, K. Okita, T. Nakazawa, Infect. Immun. 66 (1998) 4832.
[5] E. Rokita, A. Makristathis, E. Presterl, M.L. Rotter, A.M. Hirschl, J. Infect. Dis. 178 (1998) 1521.
[6] L.-T. Hu, H.L.T. Mobley, Infect. Immun. 58 (1990) 992.
[7] H.L.T. Mobley, M.D. Island, R.P. Hausinger, Microbiol. Rev. 59 (1995) 451.
[8] P.R. Hawtin, A.R. Stacey, D.G. Newell, J. Gen. Microbiol. 136 (1990) 1995.

[9] D.J. Evans, D.G. Evans, S.S. Kirkpatrick, D.Y. Graham, Microb. Pathog. 10 (1991) 15.
[10] P.K. Ng, J.L. Lundblad, G. Mitra, Sep. Sci. 5 (1976) 499.
[11] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[12] U.K. Laemmli, Nature 277 (1970) 680.
[13] H. Towbin, T. Staehelin, J. Gordon, Proc. Natl. Acad. Sci. USA 76 (1979) 4350.
[14] O. Salinovich, R.C. Montelaro, Anal. Biochem. 156 (1986) 341.
[15] E. Harlow, D. Lane, Antibodies – A Laboratory Manual, CSH Laboratory Press, Cold Spring Harbor, 1988.
[16] S.H. Phadnis, M.H. Parlow, M. Levy, D. Ilver, C.M. Caulkins, J.B. Connors, B.E. Dunn, Infect. Immun. 64 (1996) 905.
[17] A. Vanet, A. Labigne, Infect. Immun. 66 (1998) 1023.
[18] J.W. Austin, P. Doig, M. Stewart, T.J. Trust, J. Bacteriol. 173 (1991) 5663.